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(54) Title: RIBOSOME INACTIVATING PROTEINS EXTRACTED FROM SEEDS OF SAPONARIA OCYMOIDES AND VACCARIA PYRAMIDATA, THEIR PREPARATION AND IMMUNOTOXINS CONTAINING THEM			
(57) Abstract			
New proteins extracted from the seeds of Saponaria ocymoides and Vaccaria pyramidata respectively, which are able to inactivate ribosome proteins, their preparation and immunotoxins containing such proteins and an antibody, or other suitable carriers, useful in the treatment of tumors are described.			

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RIBOSOME INACTIVATING PROTEINS EXTRACTED FROM SEEDS OF SAPONARIA OCYMOIDES AND VACCARIA PYRAMIDATA, THEIR PREPARATION AND IMMUNOTOXINS CONTAINING THEM.

Field of the invention

The present invention refers to new proteins extracted from the
5 seeds of Saponaria ocymoides and Vaccaria pyramidata respectively,
which are able to inactivate ribosome proteins, their preparation
and immunotoxins (immunoconjugates) containing such proteins and an
antibody, or other suitable carriers, useful in the treatment of
tumors.

10 State of the art

It is known that many plant tissues contain proteins which are able
to inhibit the protein synthesis by inactivating the eukaryotic
ribosomes. These proteins, usually named RIP (Ribosome Inactivating
Proteins), are normally present as monomers having a molecular
15 weight of about 30 kDa (RIP type-1) and have an isoelectric point
which is often strongly basic.

Considering the fact that all the toxins used up to now induce an
immunological response in the patients, the identification and
purification of new RIP proteins is extremely important for
20 therapeutical application.

Moreover it is known that, with the aim of obtaining molecules which
are selectively cytotoxic, many of the known RIP were linked to
molecular vectors able of carrying them on specific cell populations
constituting the wanted target. Normally to prepare compounds having

a specific cytotoxic action monoclonal antibodies are used as protein vectors (immunotoxins), anyhow also hormones, Growth Factors and lectins were used as vectors in the therapeutical treatment of tumors.

- 5 For the therapeutical application the link between antibody and toxin should be strong enough to remain stable during the passage of the immunotoxin in the tissues up to the target cell and should be broken once inside the cell in order to develop its inactivating action.
- 10 Monoclonal antibodies specific for EGF-r (Epidermal Growth Factor Receptor) were used in the art to construct immunoconjugates capable of reducing the metastatic potential of tumoral cells transplanted in athymic mice.
- 15 Since EGF-R is expressed also by healthy cells it can not be considered qualitatively tumor-specific but an abnormal quantity can be exploited for the wanted purposes. Therefore in order to take advantage of the quantitative difference of expression of EGF-R between healthy and tumoral cells it is necessary to provide reagents capable of reacting selectively only with cells showing an
- 20 high expression of the factor.

Description of the figures

Figure 1 shows the elution profile of the protein obtained from Saponaria ocymoides by ion exchange chromatography on CM-Sepharose column (81.0 cm x 20 cm), prepared with sodium-phosphate buffer 5 mM (pH 7) eluted with a linear gradient 0 - 0.3 M NaCl in the same

buffer.

In the abscissa the elution volumes are reported while in ordinate the inhibition of the protein synthesis (%) is shown.

The line —— is the absorbance, the line —□—— shows the
5 protein inhibition (%) in respect of control.

Figure 2 shows the elution profile of the protein obtained from Vaccaria pyramidata by ion exchange chromatography on CM-Sepharose column (1.6 cm x 20 cm), prepared with sodium-phosphate buffer 5 mM (pH 7) eluted with a linear gradient 0.05 - 0.3 M NaCl in the same
10 buffer.

In the abscissa the elution volumes are reported while in ordinate the inhibition of the protein synthesis (%) is shown.

The line —— is the absorbance, the line —□—— shows the protein inhibition (%) in respect of control.

15 Figure 3 shows the elution profile of Piramidatine purified by reverse phase HPLC.

Figure 4 shows the elution profile of Ocymoidine purified by reverse phase HPLC. Figure 5 shows the electrophoresis SDS-PAGE of the immunoconjugates in non-reducing environment (Phast System,
20 Pharmacia; Phastgel Gradient 4 - 15%)

Figure 6 shows the inhibition of the protein synthesis on a lysate of rabbit reticulocytes by immunoconjugates MGR-6-Piramidatine (A) and MGR-6-Ocymoidine (B).

On the ordinate the inhibition in % is reported and on the abscissa

the toxin concentration.

Figure 7 shows the inhibition of the protein synthesis on a lysate of rabbit reticulocytes by immunoconjugates Mint5-Piramidatine (A) and Mint5 - Ocyroidine (B).

5 On the ordinate the inhibition in % is reported and on the abscissa the toxin concentration.

- SEQ ID N0:1 shows the nucleotide and amino acid sequence of the variable region of the heavy chain of antibody Mint5. The nucleotide sequences of eight codons at the end 5' and 11 codons at the end 3' 10 are imposed by the primers used in the amplification of the cDNA by PCR. The primers were chosen according to the work of Orlando et al., PNAS, 86:3833-3837 (1989). The sequences reported as: CDR1, CDR2 and CDR3 (CDR = complementary determining region) refer to the ioper-variable regions.

15 - SEQ ID N0:2 shows the amino acid sequence (deduced from the nucleotide sequence of SEQ ID N0:1) of the variable region of the heavy chain of antibody Mint5

- SEQ ID N0:3 shows the N-terminal fragment of the amino acid sequence of the variable region of the heavy chain. Such sequence 20 was determined by Edman's degradation. Only the amino acids 1, 3, 5 and 6 are different in respect of the amino acid chain deduced and reported under SEQ ID N0:2. The amino acids reported as Xaa were not identified correctly.

- SEQ ID N0: 4 shows the nucleotide and amino acid sequence of the 25 variable region of the light chain of antibody Mint5. The nucleotide

sequence of eight codons (24 nucleotide) at the 5' and 3' ends were imposed by the primers used in the amplification of cDNA by PCR. The sequences indicated with: CDR1, CDR2 and CDR3 refer to the ipervariable regions.

- 5 - SEQ ID NO:5 shows the amino acid sequence (deduced from the nucleotide sequence of SEQ ID NO:4) of the varable region of the light chain of the antibody Mint5.
- SEQ ID NO:6 shows the N-terminal fragment of the amino acid sequence of the variable region of the light chain of antibody
- 10 Mint5. Such sequence was determined by Edman's degradation. Only the amino acids 3 and 8 are different in respect of the amino acid chain deduced and reported under SEQ ID NO:5.
- SEQ ID NO:7 shows the amino acid sequence of the RIP obtained from Saponaria ocymoides.
- 15 - SEQ ID NO:8 shows the sequence from amino acid 38 to 73 of the RIP obtained from Vaccaria pyramidata.
- SEQ ID NO:9 shows the sequence from amino acid 112 to 123 of the RIP obtained from Vaccaria pyramidata.
- SEQ ID NO:10 shows the sequence from amino acid 235 to 258 of the
- 20 RIP obtained from Vaccaria pyramidata.
- SEQ ID NO:11 shows the sequence from 265 to 277 of the RIP obtained from Vaccaria pyramidata.

Detailed description of the invention

It is an object of the present invention new RIP proteins extracted

from Saponaria ocymoides and Vaccaria pyramidata.

The invention refers also to immunoconjugates formed by the above said proteins and monoclonal antibodies, more particularly the antibodies are antibodies specific against EGF-R (Mint5) and p185

5 HER2/neu (MGR-6). The present invention refers also to the use of the immunoconjugates for the preparation of pharmaceutical compositions useful in the therapy of tumors, autoimmunity, infections, antiviral and transplant rejection.

The present invention refers also to a method for the treatment of
10 tumors wherein a therapeutically active amount of the immunoconjugates according to the invention is administered to the patient.

The RIP according to the invention are hereinafter indicated as
15 Ocymoidine (the one extracted from Saponaria ocymoide) and Piramidatine (the one extracted from Vaccaria pyramidata).

The Rip according to the invention were purified through a method comprising, in sequence: ion exchange chromatography on S-Sepharose and CM-Sepharose followed by hydrophobic interaction chromatography on Phenyl-Sepharose (Ocymoidine) and filtration on Amicon PM10
20 membrane (Piramidatine).

Both proteins have a purity > 98% by reverse phase HPLC.

The proteins have a molecular weight (in SDS-PAGE) of about 30,000 Da (Ocymoidine) and about 24,000 Da (Piramidatine) and a strongly basic isoelectric point (about 9.5). Their amino acid composition
25 was determined.

The purified proteins show an inhibition of 50% (IC_{50}) of the protein synthesis in an acellular system of rabbit reticulocyte lysate in concentration of 1 -2 ng/ml ($IC_{50} < 10^{-10} M$).

As above said the present invention refers also to the
5 immunoconjugates formed by the above said proteins Ozymoidine and Piramidatine with antibodies.

In particular are described immunoconjugates formed by monoclonal antibodies and more in particular antibodies specific against EGF-R and p185HER2/neu, such monoclonal antibodies are hereinafter
10 indicated as Mint5 and MGR6 respectively.

Mint5 is a monoclonal antibody specific for EGF-R obtained through immunization of mice with the cell line A431 (Epidermoid carcinoma cell line over-expressing EGF receptors).

Mint5 was deposited with the DSM (Deutches Sammlung von
15 Mikroorganismen und Zellkulturen GmbH) under the number ACC2150.

Mint5 is able of discriminating between cells having normal and abnormally high expression of EGF-R. The identification threshold of Mint5 in immunocytochemistry tests is 5×10^4 receptor/cell ($10 \mu m$ diameter). Mint5 can inhibit the linking of EGF-R to the receptor on
20 target cells and conversely its bonding is inhibited by EGF. Therefore Mint5 can recognize in the receptor the linking *situ* of EGF.

Mint5 inhibits the growth of cells having high levels of EGF-R expression in vivo and in vitro but has no effect on cells

expressing normal levels of receptors.

After its binding to A431 cells, Mint5 easily enters the cell and is able to inhibit the growth of tumoral cells in transplanted athymic mice.

5 In the same way as Mint5, also MGR6 [described by Centis F. et al. Hybridoma 11(3): 267 - 276 (1992)] is able to identify a protein (p185 HER2/neu) expressed at high level on tumoral cells. This protein is the product of gene c-erbB2 expressed also in normal cells and present in chromosoma 17 codifying a protein highly
10 homologous, but different, to EGF-R.

In normal breast cells c-erbB2 is present as a single gene pair but is amplified 25 - 30% times in primary breast tumors.

MGR6 is a monoclonal antibody (IgG2/K) obtained by immunization of BALB/c mice with cells of lung carcinoma CALU3 which overexpress
15 p185 HER2/neu.

Therefore MGR6, being able of discriminating between cells having normal and abnormally high values of expression of p185 HER2/neu and being able to enter easily the cell, is a useful vector for toxin in the antitumor therapy. The immunoconjugates according to the
20 invention were prepared according to the method described by Thorpe et al.: "Monoclonal Antibody - Toxin conjugates: Aiming the magic bullet" in Monoclonal Antibodies in Clinical Medicine, Academic Press, pp.168 - 190 (1982).

This method is based on the use of hetero-bifunctional reagents as
25 N-succinimidyl-3-(2-pyridylthio)-propionate (SPDP), 2-iminothiolane

(Traut reagent), S-acetyl-mercaptosuccinic anhydride (SAMSA), carbodiimide or glutaraldehyde or the like. These reagents are used according to standard methods, in particular 2-iminothiolane is used to activate the antibody and the toxin which will be thereafter 5 conjugated by a disulfide bridge.

Immunoconjugates prepared with this method were previously used in clinical studies of the treatment of Hodgkin's lymphoma resistant to traditional treatment, with substantial remission [Falini et al. Response of refractory Hodgkin's disease to monoclonal anti-CD30 10 imunotoxin; Lancet 339: 1195-1196 (1992)].

In order to increase the stability of the compounds according to the invention their preparation by recombination-techniques were investigated.

Many researchers produced fusion protein in a single polypeptide 15 chain wherein the gene codifying the variable regions of the antibody are linked by a linker-sequence of DNA to the codifying gene for the toxin. The advantage of these molecules, a part from their chemical stability, are a consequence of their little dimension which let foresee a good pharmacokinetic and a reduced 20 immunogenicity in respect of chemical immunoconjugates. Moreover small toxins can enter more easily the tissues and therefore be more efficient against solid tumors.

Example

Preparation and purification of Pirimidatine and Ozymoidine

Vaccaria pyrimidata and Saponaria ocymoides seeds are homogenized with an Ultraturrax homogenizer in 8 volumes of cold PBS buffer (pH 7.15) (0.14 M NaCl/5 mM sodium phosphate). The suspension is extracted during 12 hours at 4°C under magnetic stirring, filtered and clarified by centrifugation at 30.000 rpm for 30 minutes at 4°C.

5 The raw extracts are acidified with glacial acetic acid up to pH 4.0 - 4.1 and centrifuged as above said.

The supernatants are applied to a S-Sepharose column (15 x 2 cm) prepared with 20 mM sodium acetate (ph 4.5). The column is washed with one volume of the above said buffer and thereafter with sodium-phosphate 5 mM (pH 7.15). Proteins were eluted with NaCl 1 M in the same buffer and dialyzed against sodium-phosphate buffer 5 mM (pH 7.15) (Pirimidatine) or against the same buffer containing 50' mM NaCl (Ocymoidine) at 4°C.

10 15 After centrifugation as above said, in order to recover the precipitate, the protein solution is applied to a CM-Sepharose FF column (22 x 1.6 cm) prepared with the corresponding dialysis buffer; the column is washed with the same buffer up to an absorbance lower than 0.1.

20 25 The elution of the adsorbed proteins is performed with 240 ml linear gradient from 0 to 300 mM of NaCl for Pirimidatine (see Fig. 1) and 1 l linear gradient from 50 to 300 mM for Ocymoidine (see Fig. 2).

The fractions inhibiting the protein synthesis in a lysate of rabbit reticulocyte [according to Stirpe et al., 1983, Biochem. J.: 216,

25 617 - 625; Allen e Scweet, 1962, J. Biol. Chem. 237, 760 - 7] are

pooled and dialyzed against water.

To eliminate low molecular weight contaminants the solution of RIP from Vaccaria pyramidata was concentrated and diluted at least 10 times on an Amicon PM 10^R membrane, and finally concentrated. The 5 solution from Saponaria ocymoides was purified by hydrophobic interaction chromatography.

The RIP solubilised in 30% (w/v) ammonium-sulphate (pH 7.2) was loaded on a Phenyl-Sepharose^R column (10 x 1.6 cm) prepared with the same buffer. The column was washed up to an absorbance (A_{280}) 10 lower than 0.05 and the adsorbed protein was eluted with PBS. The purification scheme is reported in Tables 1 and 2 together with yields and activities.

Physicochemical properties of Pirimidatine and Ocyroidine

Molecular weight was determined by electrophoresis and gel-15 filtration. The method according to Laemmli for SDS-PAGE was used with the following molecular weight markers: cytochrome c (12,300), myoglobin (17,200), carbonic anhydrase (30,000), ovalbumin (43,000), BSA (66,250) and ovotransferrin (76-78,000).

Gel-filtration was performed by HPLC with a column TSK-gel G3000SW, 20 prepared with buffer (ph 6.7) 0.1 M sodium sulphate, 0.1 M sodium phosphate and calibrated with ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000) and BSA (67,000).

The molecular weight determined by SDS-PAGE analysis is about 30,000 for Ocyroidine and about 24,000 for Pirimidatine.

The two proteins are 99% homogeneous and by gel-filtration show a molecular weight of about 30,000 (Ocymoidine) and about 29,000 (Piramidatine).

5 The analysis by reverse phase chromatography on a VYDAC protein C₄^R column (25 cm x 4.6 mm) confirmed the high purity of the two proteins so prepared (> 98%) (see Fig. 4 and 5).

The isoelectric point (pI) and the amino acid composition of the proteins were determined as described by Falasca et al. (1982). Biochem. J. 207, 505-509. The isoelectric point is strongly basic
10 (about 9.5).

The physicochemical properties are reported in Tables 3 and 4.

Biological properties of Piramidatine and Ocymoidine

Protein synthesis inhibition

15 The protein synthesis in a cell-free system was measured as described by Stirpe et al. (see above) with a rabbit reticulocyte lysate according to Allen and Schweet (see above) or by polymerisation of phenylalanine started by Poly(U) with purified ribosomes from rat liver [Montanaro et al.: Biochem. J. 176, 265 - 275 (1978)].

20 The IC₅₀ (concentration giving 50% inhibition) was calculated by linear regression.

In all cases all the IC₅₀ were inferior to 10⁻¹⁰ M (see Tables 1 and 2) and similar to those already described for other RIPS.

Modification of ribosomal RNA

25 The action of the Rips on rRNA was investigated as described by Endo

et al. [J. Biol. Chem.: 262, 5908 - 5912 (1987)]. Samples (0.01 ml) of reticulocytes lysate were incubated in the presence of 1 µg/ml of RIP for 20 minutes at 37°C. The reaction was stopped by addition of 0.5% SDS. The RNA was extracted with phenol and precipitated with 5 ethanol; in both cases, for the analysis and for the purification of the fragments, the RNA was submitted to electrophoresis on 5% polyacrylammide-gel and the gel was stained with silver as described by Stirpe et al., 1988, Nucleic Acid Res. 16, 1349 - 1357.

Both proteins damaged ribosomal RNA in the same way as ricin and 10 other RIP, as indicated by the fragment which can be obtained after treating with aniline RNA extracted from ribosomes incubated with the RIPs.

The amount of adenine produced by treated ribosomes was determined by reverse phase HPLC on Spherisorb S5 ODS2 (C18)^R column (250 x 4.6 15 mm) as described by Barbieri et al. 1992, Biochem. J. 286, 1 - 4.

The amount of adenine produced was not more than 1 mole for 1 mole of ribosomes (for example 0.82 mol/mol for ribosomes treated with Piramidatine; 0.79 mol/mol for ribosomes treated with Ocymoidine).

Activation of Piramidatine and Ocymoidine

20 The RIPs were solved up to a concentration of about 3 mg/ml in 50 mM sodium-borate buffer (pH 9). To these solutions traces of the RIPs marked with ¹²⁵I (for a total of 10⁶ cpm) were added. After centrifugation to remove corpuscular material 2-imminothiolane (2-IT) was added up to a final concentration of 1 - 2 mM.

2-IT was solved in 50 mM sodium-borate buffer (pH 9) immediately before use.

After 60' at 28°C solid glycine was added up to a final concentration of 200 mM and after 30' Ellman's reagent, solved in 50

5 µl of dimethylformamide immediately before use, was added up to a final concentration of 2.5 mM.

After 30' incubation at 38°C the sample was applied to a Sephadex G25 Coarse^R (25 x 1.6 cm) prepared and eluted with PBS.

The protein peak was collected and the activation ratio was 10 calculated on a little amount of sample diluted 1:5 with PBS after measuring the absorbance at 280 nm and 412 nm and after addition of 1/10 (v/v) of freshly prepared solution of 2-mercaptopethanol 0.22 M.

Activation of antibodies

15 The monoclonal antibodies Mint5 and MGR-6 in a concentration of 1 - 6 mg/ml were reacted respectively with 2-IT 0.3 - 0.35 mM, following the above said procedure.

Conjugation

The RIP are concentrated under nitrogen using an Amicon concentrator and reduced adding 1/10 in volume of 2-mercaptopethanol 0.22 M. The 20 reduced toxins were loaded on a Sephadex G25 Coarse^R (25 x 1.6 cm).

The protein peak was collected in the concentrator wherein the antibodies were reacting. The reaction mixture was concentrated 4 times under nitrogen, incubated for 20 h at room temperature. The mixture was purified on a Sephadryl S-200 HR^R column (96 x 2.2 cm) 25 prepared and diluted with PBS.

The radioactivity was confronted with the elution profile at 280 nm and the various components purified from the mixture were identified.

5 The first to elute is the high molecular weight conjugate (HMW), the second the conjugate at low molecular weight (LMW), therafter, in the following order, elute: trimeric RIP (when present), dimeric RIP and non-reacted RIP.

10 The non-reacted antibodies elute in the last part of the peak LMW having an absorbance of 200 nm. This is shown in the radioactive profile under the UV peak.

The LMW fractions are collected together and the ratio RIP/antibody was calculated for the absorbance at 280 nm and radioactivity of the conjugate LMW and of the non-reacted RIP.

15 The average conjugation ratio RIP/antibody is comprised between 0.86 and 1.95.

Following the same procedure the following immunoconjugates were prepared:

MGR-6 - Piramidatine

MGR-6 - Ozymoidine

20 Mint5 - Piramidatine

Mint5 - Ozymoidine

The conjugation conditions for obtaining the immunotoxins are shown in Table 5.

SDS-PAGE: Control of the antibodies activation

The conjugation degree of Mint5 and MGR-6 with Piramidatine and Ozymoidine was controlled by SDS-PAGE analysis. As shown in Figure 5 the MGR-6 antibodies (position 1) and Mint5 (position 6) migrate as a single band of about 155 KDa. The corresponding conjugates with 5 piramidatine and ozymoidine (positions 2 - 5) show multiple bands corresponding to the different degrees of conjugation.

Biological properties of the conjugates

Inhibition of protein synthesis in lysate of rabbit reticulocytes

The immunotoxins obtained according to Table 5 were tested for their 10 capacity of inhibiting the protein synthesis in a system of lysate of rabbit reticulocytes. Figures 6 and 7 show that the immunoconjugates MGR-6-Piramiditine, MGR-6-Ozymoidine, Mint5-Piramiditine and Mint5-Ozymoidine in a concentration of 10^{-9} M (calculated in respect of the toxin content in the immunoconjugate) 15 can inhibit 50% the protein synthesis.

Binding of the immunoconjugates on the target cells

The capacity of the immunoconjugates to bind specifically to the target cells was demonstrated by radioimmunological test. A431 cells (cell line expressing high levels of EGF-R and therefore target for 20 the antibody Mint5) or SKBR3 cells (cell line expressing high levels of p185 HER2/neu and therefore target for the antibody MGR-6) were fixed on a plate with 96 wells and incubated with the antibodies or the immunoconjugates. The binding was demonstrated with a secondary antibody anti-murine immunoglobulins marked with ^{125}I . The results 25 shown in Table 6 show that the immunoconjugates bind specifically to

corresponding cell targets.

Inhibition of cell proliferation and inhibition of protein synthesis
on target cells

The inhibition of cell proliferation was performed according to
5 Table 7, following two different procedures reported as protocol A
and protocol B, and the results of the toxicity tests are shown in
Table 9.

The inhibition of protein synthesis was performed according to Table
8, following two different procedures reported as protocol A and
10 protocol B, and the results of the toxicity tests are shown in
Table 10.

Tables 7 and 9 show that when, protocol A is performed, an increase
of 100 times of the toxic activity, when the toxin is bound to the
antibody, is observed, whilst when protocol B is performed an
15 increase of 1000 times is observed.

Tables 8 and 10 show that when protocol A is performed, an increase
of 1000 times of the toxic activity, when the toxin is bound to the
antibody, is observed, whilst when protocol B is performed an
increase of 10000 times is observed.

TABLE 1

ACTIVITY OF RIP FROM SEEDS OF VACCARIA PYRAMIDATA (25g)

	Total protein mg	IC ₅₀ ng/ml	Specific activity U/mg $\times 10^3$	Total activity U/ 10^6	Activity (yield) %
Raw material	939.1	148.0	6.76	6.35	100
Acid treatment	821.5	151.0	6.62	5.44	85
S-Seph	160.5	33.1	30.21	4.85	76
CM-Seph	9.2	2.5	401.61	3.67	58

TABLE 2

ACTIVITY OF RIP FROM SEEDS OF SAPONARIA OCYMOIDES (25g)

	Total protein mg	IC ₅₀ ng/ml	Specific activity U/mg $\times 10^3$	Total activity U/ 10^6	Activity (yield) %
Raw material	698.7	24.8	40.73	28.46	100
Acid treatment	487.2	20.9	47.87	23.32	82
S-Seph	176.8	5.0	199.60	35.28	124
CM-Seph	44.9	1.9	537.63	24.16	85
Phenyl-Seph	31.0	1.4	740.74	22.96	81

U = Toxin concentration inhibiting (50%) the protein synthesis in 1 ml of the system of rabbit reticulocytes lisate in 5' at 28°C

TABLE 3

Physicochemical properties of Piramidatine

M_r from gel filtration	28.000
M_r from SDS-PAGE	24.500
pI	≥ 9.5
$A_{1cm, 280}$	6.42

Amino acid	Composition (mol/mol)
Lysine	12.1
Histidine	----
Arginine	14.8
Aspartic acid/Asparagine	22.0
Threonine *	12.0
Serine *	9.5
Glutamic acid/Glutamine	19.0
Proline	6.9
Glycine	13.9
Alanine	13.4
Cysteine	----
Valine	12.3
Methionine	2.1
Isoleucine	11.9
Leucine	15.1
Tyrosine *	10.1
Phenylalanine	8.3
Tryptophan	N.D.
Total residues	183.4

N.D. = value not determinable

* The value obtained from hydrolysis at 24, 48 and 72 hours
were extrapolated at time zero

 M_r = relative molecular weight

A = Absorbance

pI = isoelectric point

TABLE 4

Physicochemical properties of Ocytoidine

M_r from gel filtration 30.200

M_r from SDS-PAGE 30.200

pI ≥ 9.5

$A_{1cm, 280}^{1\%}$ 4.80

Amino acid	Composition (mol/mol)
Lysine	22.1
Histidine	2.7
Arginine	13.5
Aspartic acid/Asparagine	33.2
Threonine *	12.7
Serine *	13.3
Glutamic acid/Glutamine	19.0
Proline	7.8
Glycine	13.3
Alanine	20.3
Cysteine	---
Valine	14.5
Methionine	4.8
Isoleucine	12.4
Leucine	21.1
Tyrosine *	9.0
Phenylalanine	11.9
Tryptophan	N.D.

Total residues 231.6

N.D. = value not determinable

* The value obtained from hydrolysis at 24, 48 and 72 hours were extrapolated at time zero

M_r = relative molecular weight

A = Absorbance

pI = isoelectric point

TABLE 5

Immunoconjugate	Antibody		RIP		Immunoconjugate			
	ZIT (mM)	conjugated groups mol/ mol	ZIT (mM)	conjugated groups mol/ mol	RIP/ Ab mol/ mol	Ab μg/ml Mx10 (-7)	RIP μg/ml Mx10 (-7)	
MGR6- Piramidatine	0.30	2.22	1.00	0.81	0.86	92.0 6.13	12.7 5.28	
MGR6- Ocymoidine	0.30	2.35	1.00	0.76	1.63	97.7 6.51	31.8 10.6	
Mint5- Piramidatine	0.35	2.40	1.00	0.80	1.95	110 7.32	34.4 14.3	
Mint5- Ocymoidine	0.35	2.09	1.60	0.79	1.82	141 9.40	51.2 17.0	

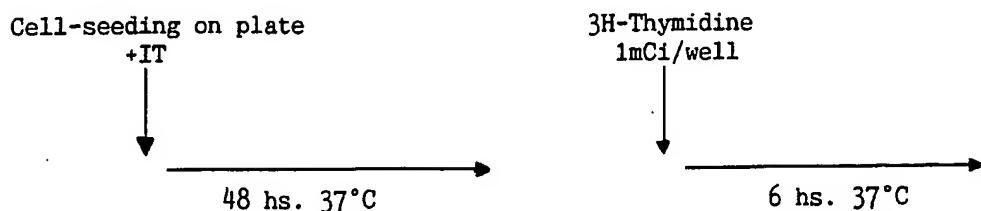
TABLE 6

	% binding	
	SKBR3	A431
Mint5	-	100
MGR6	100	-
Mint5 - Ocytidine	9.38	78.5
Mint5 - Piramidatine	18.8	110.0
MGR6 - Ocytidine	87.5	15.3
MGR6 - Piramidatine	105.2	13.0

TABLE 7

INHIBITION OF CELL PROLIFERATION: IC₅₀

PROTOCOL A:



PROTOCOL B:

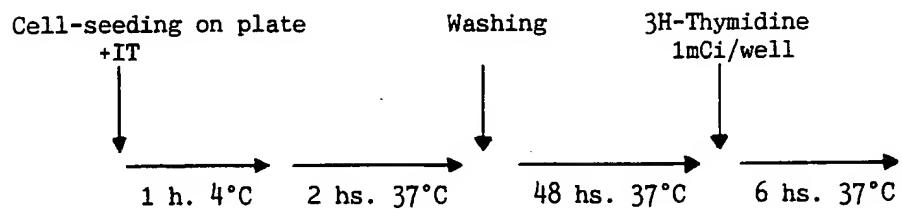
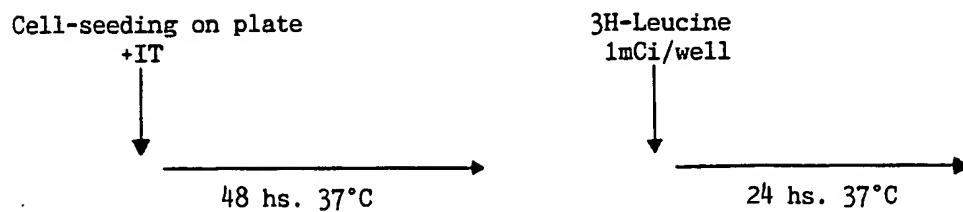


TABLE 8
INHIBITION OF PROTEIN SYNTHESIS
ON A431 CELLS

PROTOCOL A:



PROTOCOL B:

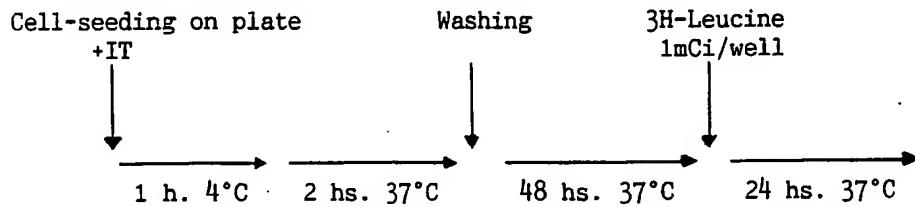


TABLE 9

INHIBITION OF CELL PROLIFERATION: IC₅₀
CELL LINE A431

Protocol	IT	TOX	RATIO
A	Mint-Ocym 1.9×10^{-12}	Ocym 2.8×10^{-10}	100
	Mint-Piram 1.3×10^{-12}	Piram 2.8×10^{-10}	100
B	Mint-Ocym 3.2×10^{-11}	Ocym 1.5×10^{-8}	1000
	Mint-Piram 4.2×10^{-11}	Piram 1.5×10^{-8}	1000

IT = Immunotoxin

TOX = Toxin

TABLE 10

INHIBITION OF PROTEIN SYNTHESIS IN CELL SYSTEM: IC₅₀
CELL LINE A431

Protocol	IT	TOX	RATIO
A	Mint-Ocym 1×10^{-11}	Ocym 2×10^{-8}	1000
	Mint-Piram 1×10^{-11}	Piram 1.5×10^{-8}	1000
B	Mint-Ocym 1×10^{-11}	Ocym 2×10^{-7}	10000
	Mint-Piram 1×10^{-11}	Piram 2×10^{-7}	10000

IT = Immunotoxin

TOX = Toxin

CLAIMS

- 1 1. Ribosome inactivating protein obtained by extraction from seeds
- 2 of Vaccaria pyramidata according to the following process:
- 3 a) seeds are homogenized with PBS (pH 7.15, 0.14 M NaCl/5 mM sodium
- 4 phosphate), centrifuged, acidified with glacial acetic acid up to pH
- 5 4.0 - 4.1 and centrifuged;
- 6 b) the supernatant of step (a) is purified with S-Sepharose column
- 7 prepared with 20 mM sodium acetate (pH 4.5), the column is washed
- 8 with one volume of the above said buffer and thereafter with 5 mM
- 9 sodium phosphate buffer (pH 7.15);
- 10 c) the protein is eluted with the same buffer containing also NaCl
- 11 1M, dialyzed against 5 mM sodium phosphate buffer (pH 7.15) at 4°C
- 12 and centrifuged;
- 13 d) the solution from step (c) is loaded on a CM-Sepharose FF column
- 14 prepared with the dialysation buffer and washed with the same buffer
- 15 up to an absorbance lower than 0.1 with 240 ml of linear gradient
- 16 from 0 to 300 mM NaCl;
- 17 e) the fractions presenting inhibiting activity on ribosome are
- 18 collected and dialyzed against water at 4°C;
- 19 f) the solution is concentrated on Amicon PM 10 membrane (at least
- 20 10 times), diluted (at least 10 times) and concentrated;
- 21 such protein having an electrophoretic mobility in SDS-PAGE as a
- 22 double band of about 24.000 Da, molecular weight by gel filtration
- 23 about 29.000 Da, isoelectric point \geq 9.5 and the following aminoacid

24 composition:

25	Lysine	12.1
26	Histidine	----
27	Arginine	14.8
28	Aspartic acid/Asparagine	22.0
29	Threonine	12.0
30	Serine	9.5
31	Glutamic acid/Glutamine	19.0
32	Proline	6.9
33	Glycine	13.9
34	Alanine	13.4
35	Cysteine	----
36	Valine	12.3
37	Methionine	2.1
38	Isoleucine	11.9
39	Leucine	15.1
40	Tyrosine	10.1
41	Phenylalanine	8.3
42	Tryptophan	N.D.

- 1 2. Ribosome inactivating protein obtained by extraction from seeds
- 2 of Saponaria ocymoides according to the following process:
 - 3 a) seeds are homogenized with PBS (pH 7.15, 0.14 M NaCl/5 mM sodium phosphate), centrifuged, acidified with glacial acetic acid up to pH
 - 4 4.0 - 4.1 and centrifuged;
 - 5 b) the supernatant of step (a) is purified with S-Sepharose column

7 prepared with 20 mM sodium acetate (pH 4.5), the column is washed
8 with one volume of the above said buffer and thereafter with 5 mM
9 sodium phosphate buffer (pH 7.15);
10 c) the protein is eluted with the same buffer containing also 1M
11 NaCl, dialyzed against 5 mM sodium phosphate buffer (pH 7.15) at 4°C
12 and centrifuged;
13 d) the solution from step (c) is loaded on a CM-Sepharose FF column
14 prepared with the dialysation buffer and washed with the same buffer
15 up to an absorbance lower than 0.1 with 1 l of linear gradient from
16 50 to 300 mM NaCl;
17 e) the fractions presenting inhibiting activity on ribosome are
18 pooled and dialyzed against water at 4°C
19 f) purification by hydrophobic interaction chromatography by
20 dissolving the product from step (e) in 30% (p/v) ammonium sulphate,
21 0.5 M NaCl in 20 mM sodium phosphate (pH 7.15) and treating with a
22 Phenyl-sepharose column prepared with the same buffer, washing up to
23 an absorbance lower than 0.5 and eluting the protein with PBS;
24 such protein having molecular weight in SDS-PAGE of 30.000 Da,
25 molecular weight by gel filtration 30.000 Da, isoelectric point ≥
26 9.5 and the following amino acid composition:
27 Lysine 22.1
28 Histidine 2.7
29 Arginine 13.5
30 Aspartic acid/Asparagine 33.2

31	Threonine	12.7
32	Serine	13.3
33	Glutamic acid/Glutamine	19.0
34	Proline	7.8
35	Glycine	13.3
36	Alanine	20.3
37	Cysteine	----
38	Valine	14.5
39	Methionine	4.8
40	Isoleucine	12.4
41	Leucine	21.1
42	Tyrosine	9.0
43	Phenylalanine	11.9
44	Tryptophan	N.D.

1 3. Process for the purification of the protein according to claim 1.

1 4. Process for the purification of the protein according to claim 2.

2 5. Conjugates of the proteins according to claim 1 and 2 with
3 hormones, lysosomes, monoclonal antibodies, Growth Factors, lectins,
4 cytokines, transferrin and peptides which are fragments of such
5 proteins.

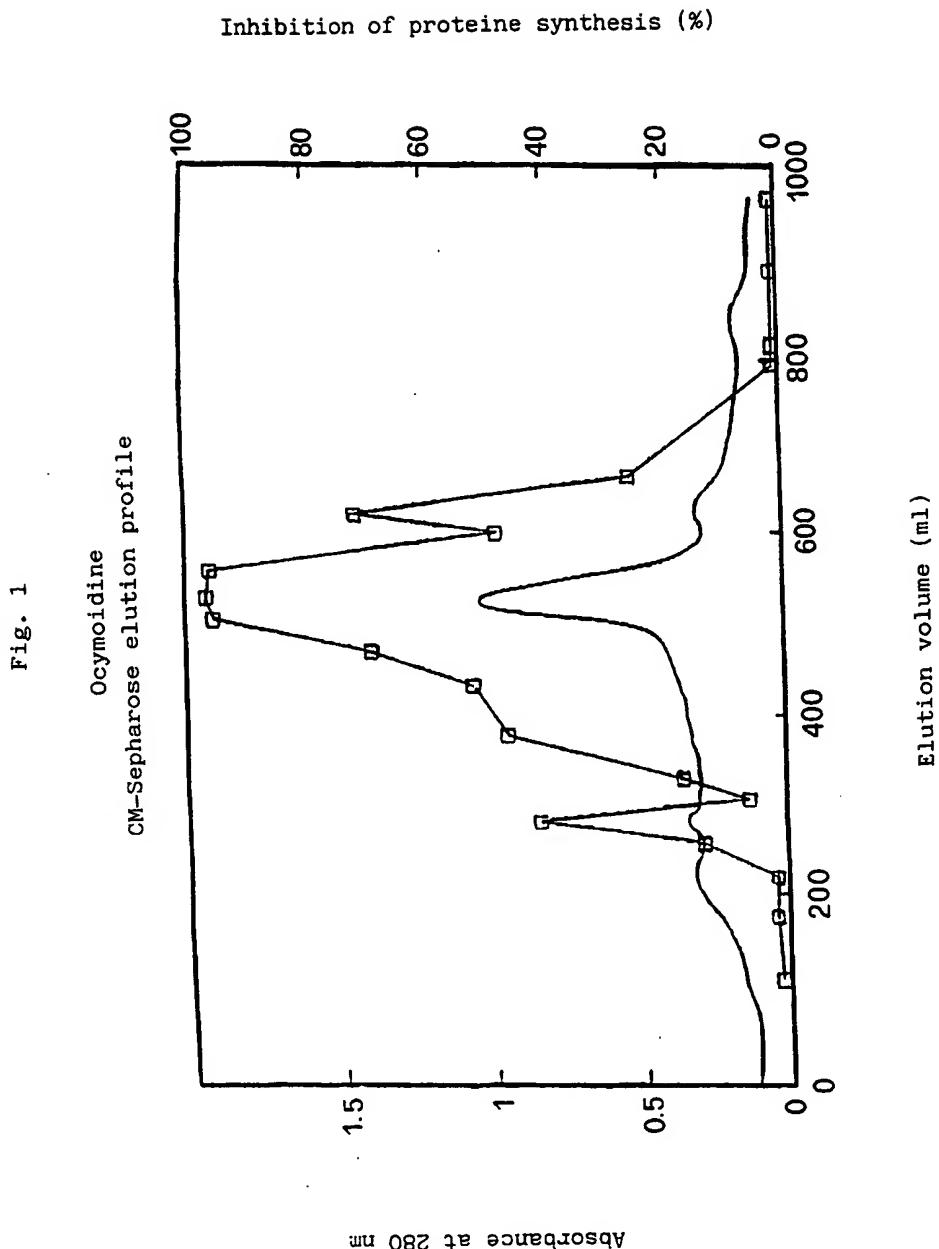
1 6. Conjugate according to claim 5 wherein the antibody is the
2 monoclonal antibody Mint5, specific for EGF-R.

1 7. Conjugate according to claim 5 wherein the antibody is the
2 monoclonal antibody MGR6, specific for HER2/neu.

1 8. Conjugates according to claim 6 and 7 obtained by chemical

- 2 conjugation or by recombination of the genes of the variable region
3 of the antibody and those codifying for the toxin.
- 1 9. Pharmaceutical compositions containing as active principle the
2 proteins according to claims 1 and 2 in combination with
3 pharmaceutically acceptable carriers.
- 1 10. Pharmaceutical compositions according to claim 9 for use as
2 anti-viral.
- 1 11. Use of the conjugates according to claims 5 - 8 for the
2 preparation of pharmaceutical compositions useful in the therapy of
3 tumors, auto-immunity, infections, antiviral therapy and transplant
4 rejection.
- 1 12. Method for the treatment of tumor characterised by the fact that
2 therapeutically appropriated amounts of conjugates according to
3 claims 5 - 8 are administered to the patient.

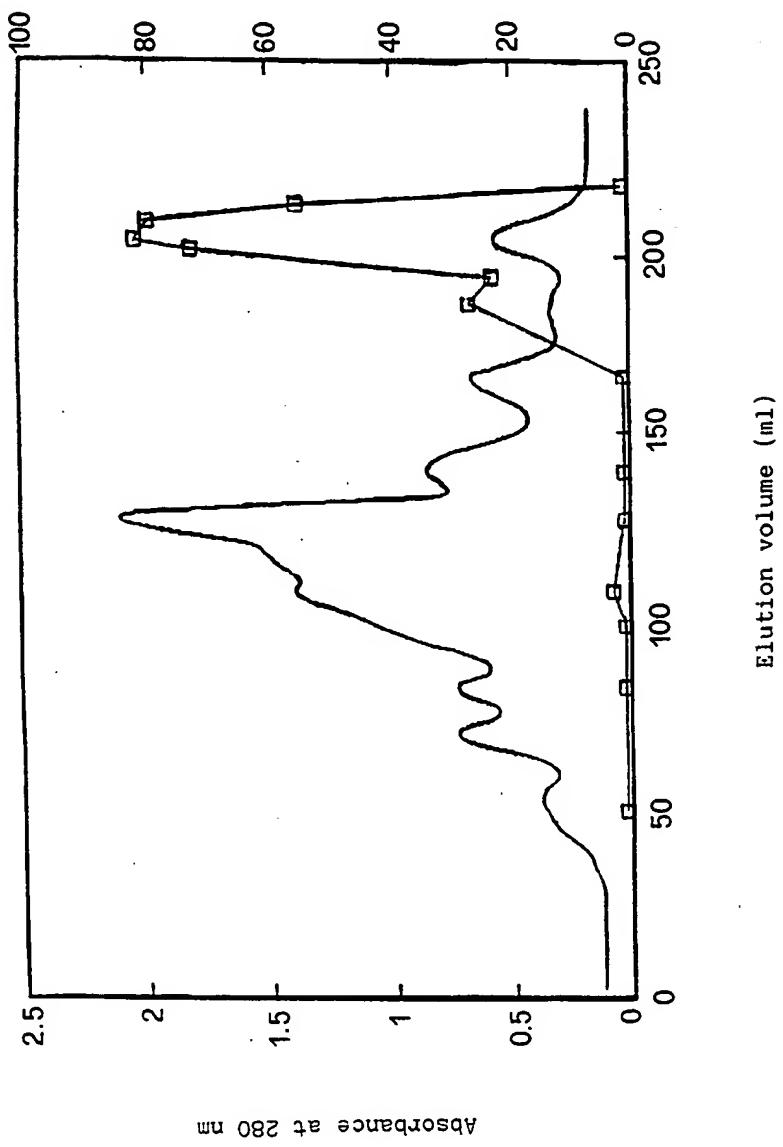
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Inhibition of protein synthesis

Fig. 2
Plramidoline
CM-Sephadose elution profile

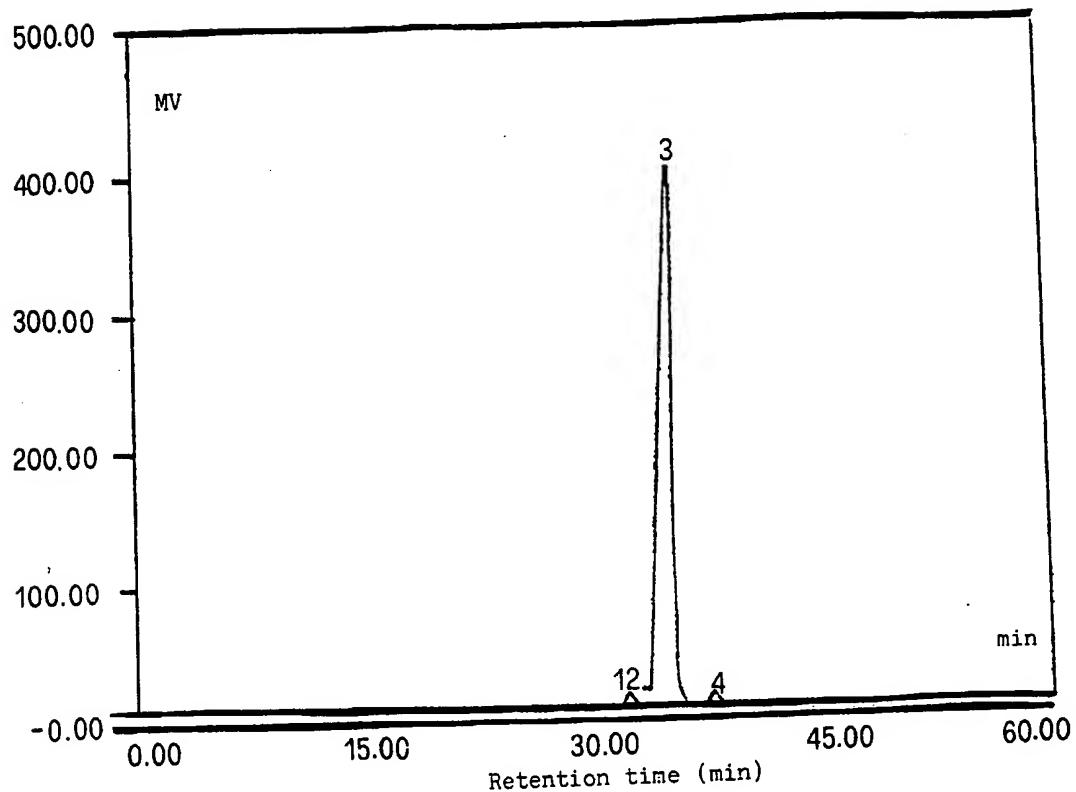


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Fig. 3

Elution profile of purified Piramidatine

Reverse phase HPLC-chromatography



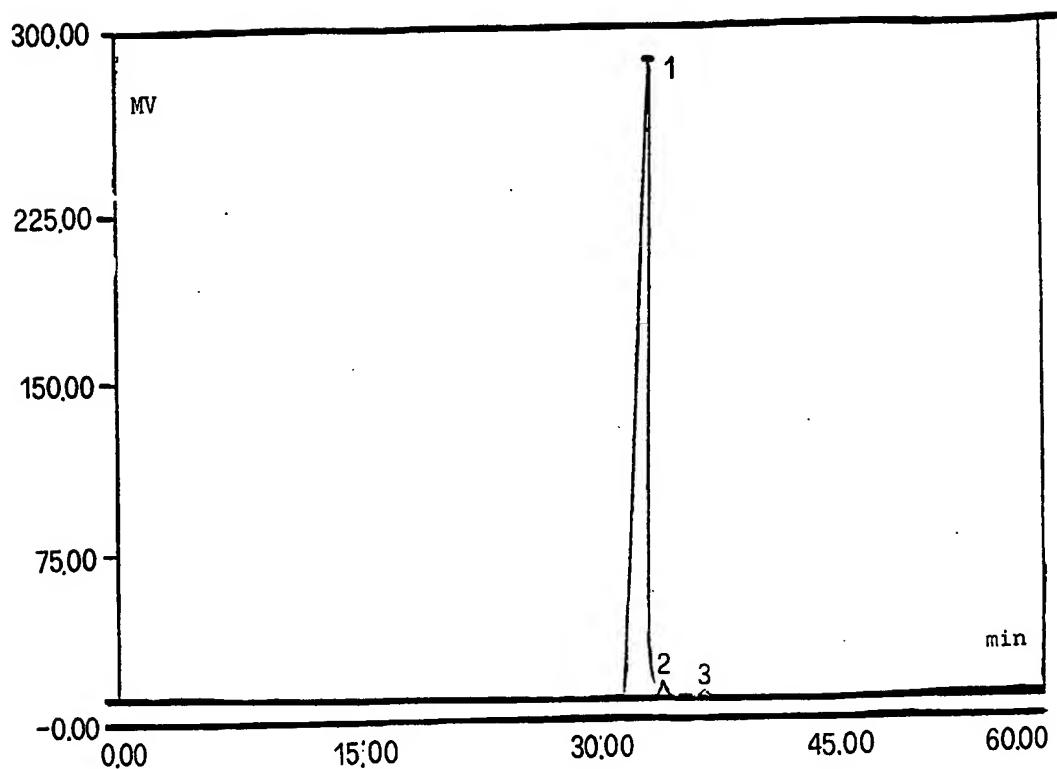
Chromatography in acetonitrile (0-70% in 50')
trifluoroacetate 0.1% buffer gradient

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Fig. 4

Elution profile of purified Ocymoidine

Reverse phase HPLC-chromatography

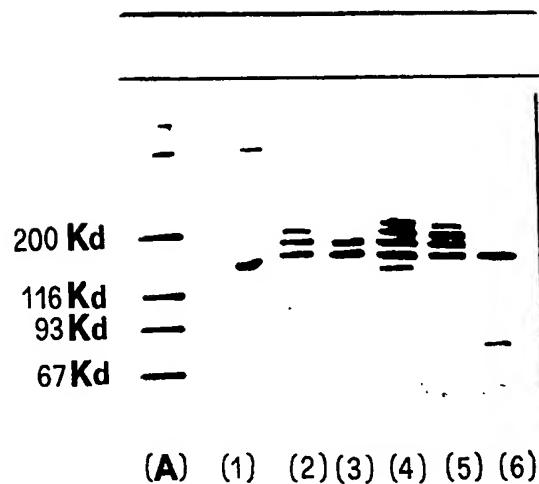


Retention time (min)

Chromatography in acetonitrile (0-70% in 50')
trifluoroacetate 0.1% buffer gradient

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Fig. 5



(A) = Reference molecular weight

(1) = Antibody MGR6

(2) = MGR6 - Ocymoidine

(3) = MGR6 - Piramidatine

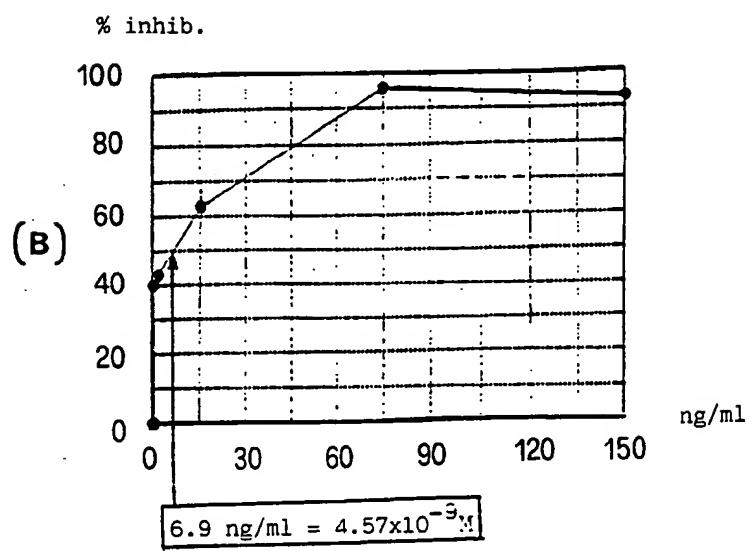
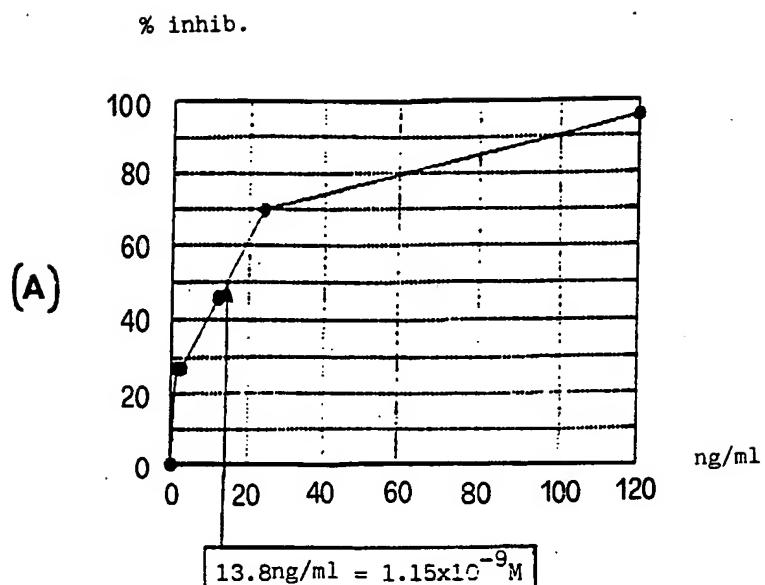
(4) = Mint5 - Ocymoidine

(5) = Mint5 - Piramidatine

(6) = Antibody Mint5

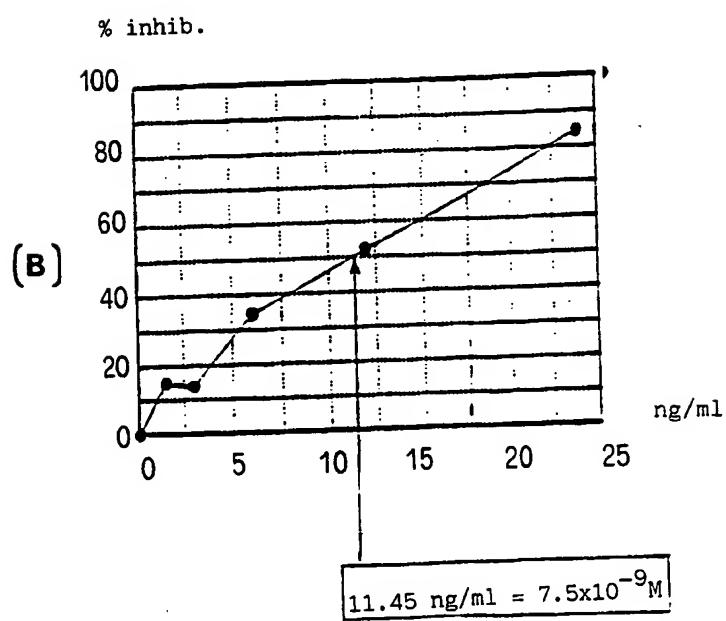
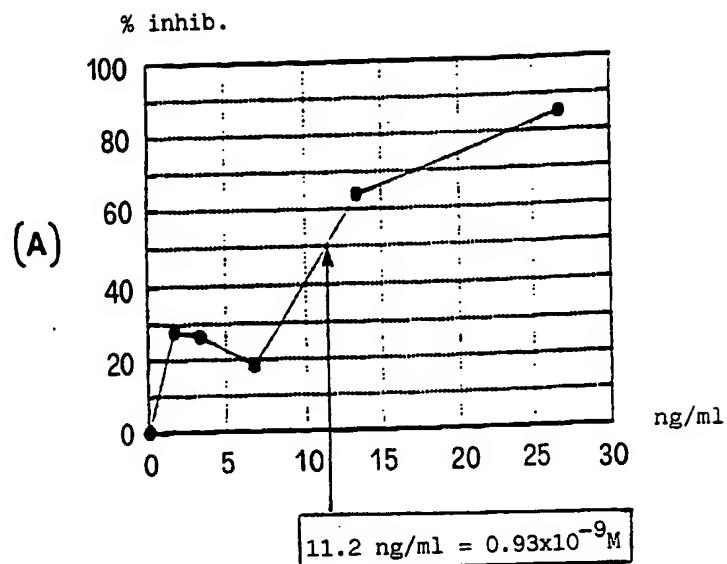
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Fig. 6



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Fig. 7



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/02969

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/415 A61K47/48 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE EUROPEAN JOURNAL OF CANCER , VOL. 27, SUPPL 3, 1991 page S57 E. TOSI ET AL 'The anti EGFR Mint5 is able to specifically target RIP alpha-sarcin cytotoxicity against relevant target cells' *see abstract 7.041 *</p> <p>---</p>	1,2,5,6, 9,11,12
A	<p>LANCET THE, vol.339, 16 May 1992, LONDON GB pages 1195 - 1196 B. FALINI ET AL 'Response of refractory Hodgkin's disease to monoclonal anti-CD30 immunotoxin' cited in the application see the whole document</p> <p>---</p> <p>-/-</p>	1,2,5,9, 11,12

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *'A' document defining the general state of the art which is not considered to be of particular relevance
- *'B' earlier document but published on or after the international filing date
- *'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *'O' document referring to an oral disclosure, use, exhibition or other means
- *'P' document published prior to the international filing date but later than the priority date claimed

- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *'&' document member of the same patent family

* 1

Date of the actual completion of the international search 20 December 1994	Date of mailing of the international search report 30.12.94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Le Correc, N

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/02969

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INTERNATIONAL JOURNAL OF CANCER, vol.55, no.1, 19 August 1993 pages 122 - 127 R. TECCE ET AL 'Characterization of cytotoxic activity of Saporin anti-GP185/HER-2 immunotoxins' see the whole document ---	1,2,5,7, 11,12
A	WO,A,90 12034 (ITALFARMACO S.P.A.) 18 October 1990 see examples 1-2 ---	1,2
A	WO,A,90 12597 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 1 November 1990 see the whole document ---	1,2,5
A	THE BIOCHEMICAL JOURNAL, vol.216, no.3, 15 December 1983 pages 617 - 625 F. STIRPE ET AL 'Ribosome-inactivating proteins from the seeds of Saponaria officinalis L. (soapwort), of Agrostemma githago L. (corn cockle) and of Asparagus officinalis L. (asparagus), and from the latex of Hura crepitans L. (sandbox tree)' ----	1-4
T	BIOCHIMICA ET BIOPHYSICA ACTA, vol.1154, no.3/4, 1993 pages 237 - 282 L. BARBIERI ET AL 'Ribosome-Inactivating Proteins from plants' -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 94/02969

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 12 is directed to a method of treatment of the human/animal body (Rule 39.1(iv)PCT) the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No

PCT/EP 94/02969

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9012034	18-10-90	AU-A-	5333490	05-11-90
		CA-A-	2050592	01-10-90
		CN-A-	1046335	24-10-90
		EP-A, B	0390040	03-10-90
		EP-A-	0465495	15-01-92
		ES-T-	2055203	16-08-94
		JP-T-	4504252	30-07-92
WO-A-9012597	01-11-90	US-A-	5191067	02-03-93
		CA-A-	2053275	28-10-90
		DE-D-	69010330	04-08-94
		DE-T-	69010330	20-10-94
		EP-A-	0470183	12-02-92
		JP-T-	4507093	10-12-92